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CHAPTER 11

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Fermented Soybean Food Products

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INTRODUCTION

Soybeans have been an important source of protein, fat, and flavor for oriental people for thousands of years. While a large variety of foods were developed from soybeans, the four most important were miso, shoyu (soy sauce), tempeh, and tofu. While the fermented products, miso and shoyu, contribute amino acids to the diet, their contribution is more in flavor than in nutrition. Having a high content of protein and fat, tofu makes a substantial contribution to nutrition. Tempeh, which is used mostly in Indonesia, has a good flavor and is also rich in both protein and fat.

The development of fermented foods, which depends on the use of rather sophisticated microbiology, was a remarkable achievement in the early history of China. There is considerable evidence (Chap. 1) that the Buddhist priests, who taught the people to avoid eating meat, were largely responsible for the development of fermented foods in the Orient. The somewhat lesser known fermented products included in this chapter are natto, hamanatto, and sufu, as well as recent work on the development of an American cheeselike product. The ancient methods of making fermented foods are changing rapidly through the introduction of modern microbiology and technology. Today, Japan has become a leader in the field of industrial microbiology.

To make some of these products, fermentation is carried out directly on cooked soybeans with a selected organism under specific conditions. In making miso and shoyu, a koji is prepared in a preliminary fermentation that is used in a second stage to ferment a combination of cooked soybeans and cereal; the cereal is usually rice when making miso and wheat when making shoyu. Koji is used also in other fermentations such as distilled spirits (shocha), sake, wine, and other beverages where it serves in place of malt.

KOJI

The word "koji" (Tamiya 1958) is an abbreviation of kabi-tacki meaning "bloom of mold." The process, like much of Japanese culture, was introduced into Japan from China about 200 A.D. Koji is a source of enzymes for converting starch into fermentable sugars and proteins into peptides and amino acids. The koji molds are grown on rice to produce amylases, which convert rice starch to fermentable sugars, so that in the second fermentation stage sugar becomes available for the yeast growing in the moromi or mash.

The microorganisms used in koji are almost always fungi of the genus *Aspergillus.* When we examine the tane koji (seed or inoculum used to make koji), we may find a great deal of variation in the purity of the inoculum. Some starters contain a mixture of mold types, including mucoraceous molds, various aspergilli and penicillia, along with an assortment of bacteria and yeasts. Obviously, these starters were not prepared under controlled conditions and contain many "weed" microorganisms. Such preparations should be avoided because of the possibility of mycotoxins. On the other hand, in modern plants making koji starters, extreme care is taken to maintain vigorous tane koji that are pure cultures containing one or more purposely mixed strains whose spores have an extremely high and rapid rate of germination (98-99%). As far as we are aware, the molds that make koji belong to the *Aspergillus oryzae* group and include *A. oryzae* and *A. sojae.* Other fungi are employed to a certain extent for the conversion of starch to fermentable sugars in the amylo-koji process including strains of *Rhizopus* according to Inui *et al.* (1965). Some of the black aspergilli in the *A. niger* group may also be used.

Molds used for koji have been extensively investigated and are known to produce a great variety of enzymes including α-amylase, proteases (3 types are known, 1 is active at alkaline, 1 is active at acid and 1 is active at neutral pH's), nucleases, sulfatases, phosphatases, transglycosidases, peptidases, acylase, ribonucleo-depolymerases, mononucleotide phosphatase, adenyl-deaminases and purine nucleosidases.

The modern koji process begins with growing a selected *A. oryzae* strain on an agar slant in pure culture. The strain is selected for its special abilities by natural selection or by induced mutation and must have the ability to sporulate luxuriantly on rice. Selection is made to give a desirable koji for a particular fermentation. Spores from the mature slant culture are used to inoculate 1-1½ kg of moist, sterile, brown rice in a wooden tray, which is also sterile. To the rice is added 2% wood ash as a source of trace elements. Addition of ash of certain trees is an important step in the process because the RNA/DNA ratio is higher. A high RNA/DNA ratio gives spores with greater viability and vigor. After five days' incubation, spores from the first tray are used to inoculate all the sterile wooden trays of the tane production plant.

The entire production area is such that it may be sterilized, and workers entering areas devoted to spore production must go through a sterile entry room dressed entirely in sterile clothing to prevent contamination. In incubation rooms, both trays and incubator are sterilized with steam between each spore run. Spores are produced at a temperature of 30° C, dried at 50° C, and stored at 15° C for sale. Purity of the inoculation is regularly determined. Spores from one or more strains are blended together and are prepared for a particular fermentation. Thus, tane koji for the miso fermentation is composed of several strains mixed together in a definite proportion: 150 gm of inoculum is equal to 1×10^{12} viable spores.

If we plate out the tane koji used in red miso fermentation, we will encounter perhaps 3 distinct morphological strains: 1 tall and light sporing with a brownish tint; a second, which is rather short, heavily sporulating and yellow green; and an intermediate form sporulating vigorously and yellow green.

The use of tane koji to prepare specific types of koji needed in fermenting soybeans will be described under the appropriate fermentations in which the various substrates are rice or wheat as well as soybeans. Besides the type of substrate used, the appearance of the koji will be different. For example, in koji prepared from rice, the mold will have converted the rice into a solid cake bound together with the mold mycelium and will be harvested just before sporulation when the conidiophores of the mold are just forming. At this stage, the maximum amount of desirable enzymes will be formed, but the molds will not have produced any undesirable odors and flavors. On the other hand, koji for shoyu, composed of roasted cracked wheat and soybean flakes at the time of harvest, will be green as a result of the sporulation of the *Aspergillus* strains.

In the older methods of making koji, wooden trays were employed but today much of the koji is made by automatic equipment in rooms in which the substrate is loaded, inoculated, turned, and harvested mechanically; the conditions of moisture, temperature, and aeration all being carefully controlled with automatic equipment. Under these conditions practically no contamination occurs and the chances for any mycotoxins or harmful bacteria being present are excluded. Incidentally, all the certified strains of molds used in Japan have been tested for the absence of toxin.

MISO

Miso is a food prepared by the fermentation of soybeans and salt with or without a cereal. Innumerable variations are possible based on the ratios of substrate, salt, length of fermentation, and aging. This account will be general in nature and follows the procedure for making red miso. For a longer account of the process, see Shibasaki and Hesseltine (1962) and Watanabe (1969).

Miso is produced in a number of countries of the Orient. In China it is called chiang. Actually, the product is produced and consumed over the whole area of Japan, China, Indochina, Indonesia, and the East Indies.

In appearance, miso is a paste resembling peanut butter in consistency and smooth in texture. But in China, chiang may be unground so that individual particles of soybeans are present. Its color varies from a light, bright yellow to a very blackish brown. Generally speaking, the darker the color the stronger the flavor. The product is typically salty and has a distinctive pleasant aroma.

Usually, miso is not consumed by itself but dissolved in water as a base for various types of soups. Often the soup contains vegetables, algae, tofu, or fish. It can serve as a seasoning for cooked meat and vegetable dishes and may be mixed with fresh vegetables such as cucumbers. Sometimes it may be added to fish. In Western culture, its counterpart is catsup. Currently, miso is made in Hawaii and is available in the United States, being packaged like sausages in plastic tubing.

Watanabe (1969) summarized the statistics concerning traditional foods in Japan. We have abstracted some of his data on the fermented foods of Japan (Table 11.1). In Table 11.2, we have also abstracted Watanabe's data on the composition and cost of fermented foods. According to him, 180,000 metric tons of soybeans and defatted soybean meal are used annually in Japanese factories for miso manufacture and an estimated additional 60,000–70,000 metric tons of soybeans are turned into various types of miso in the home. Figures on annual production of miso are shown in Table 11.3. It must be remembered that miso contains water and cereals, as well as soybeans.

Miso may be classified in various ways. The substrates used with soybeans may be rice, barley, or only soybeans. About 80% is made from rice and soybeans and the remaining 20% is of the last two types. In 1965, the various ingredients to produce 492,650 metric tons of miso were, in metric tons: soybeans, 150,181; defatted soybeans, 12,504; rice, 77,250; barley, 14,413; salt, 65,393; and corn meal, 3,842. The last is a substitute for rice. As noted, miso may be classified on the basis of color and taste of the finished product which ranges from white and light yellow to red and from sweet to salty. Rice miso is the most popular and may be divided into five types. These are white miso, light-yellow sweet miso, light-yellow salty miso and yellow-red sweet miso and yellow-red salty miso. These types of rice miso are made by varying the ratio of ingredients and soaking and cooking conditions involving varying the time, temperature, and length of fermentation and ripening. Ebine (1967), for example, collected detailed information about three types of miso which is summarized in Table 11.4. Miso can also be classified on the basis of the area where it is produced; namely, Sendai, Shinshu, etc.

TABLE 11.1

DEMAND FOR WHOLE SOYBEANS IN JAPAN

Soybeans	1964	1966	1967
		(1000 Metric Tons)	
Whole			
Miso	145	158	169
Shoyu	16	15	15
Natto	30	38	47
Defatted			
Miso	15	10	8
Shoyu	165	162	154
Total used			
Miso	160	168	177
Shoyu	181	177	169
Natto	30	38	47

TABLE 11.2

CHEMICAL COMPOSITION OF SOYBEAN FOODS

Fermented Foods	Moisture (%)	Protein (%)	Fat (%)	Soluble Carbohydrate (%)	Fiber (%)	Ash (%)	Retail Cost in 1968 (¢/100 Gm)
Miso							
Salty light	50.0	12.6	3.4	19.4	1.8	12.8	3.9
Salty red	50.0	14.0	5.0	14.3	1.9	14.8	
Soybeans	47.5	16.8	6.9	13.6	2.2	13.0	
Natto	58.5	16.5	10.0	10.1	2.3	2.6	5.5
Soybeans	12.0	34.3	17.5	26.7	4.5	5.0	

TABLE 11.3

ANNUAL PRODUCTION OF MISO

Year	Tons	Year	Tons
1956	530,078	1962	453,955
1957	520,176	1963	476,533
1958	514,974	1964	473,846
1959	505,354	1965	492,650
1960	505,086	1966	510,304
1961	482,357	1967	520,510

TABLE 11.4

COMPOSITION OF MISO IN RELATION TO TIME OF FERMENTATION AND RATIO OF SOYBEANS:RICE:SALT

Item	White Miso	Light-yellow Salty Miso	Yellow-red Salty Miso
Soybean:rice:salt	100:200:35	100:60:45	100:50:48
Duration and temperature of fermentation	2–4 days, 50° C	30 days, 30°–35° C	60 days, 30°–35° C
Color	Bright light yellow	Light yellow	Yellow red
Taste	Very sweet	Salty	Salty
NaCl (%)	5	12–13	12.5–13.5
Moisture (%)	43	48	50
Protein (%)	8	10	12
Sugar (%)	20	13	11
Shelf-life	Short	Fairly long	Long

In miso manufacture, methods differ from variety to variety, but basically the process is the same. Briefly, it involves the cleaning and cooking of soybeans; preparation of rice koji; mixing of soybeans, salt, koji, and inoculum; fermentation under anaerobic conditions; and blending and packaging of the product for market. The process is outlined on a laboratory scale in Figure 11.1

Miso manufacture is essentially two successive fermentations. The first involves the preparation of koji under aerobic conditions from strains of *A. oryzae* and *A. sojae.* The molded rice koji serves as source of enzymes and nutrients for the second fermentation. The second is an anaerobic fermentation involving yeasts and bacteria.

Preparation of Koji

Rice koji is prepared from polished rice since it is essential that mold mycelium quickly penetrate the rice kernels. Brown rice is therefore not suitable. Polished rice is soaked in cold water (about 15° C) overnight or until the moisture content is about 35%. Excess water is drained off and the swollen rice is cooked with steam at atmospheric pressure for 40–60 min, cooled to 35° C, and inoculated at the rate of 0.1% tane koji (*A. oryzae* inoculum). In modern plants the cooling and inoculation are carried out as a continuous process.

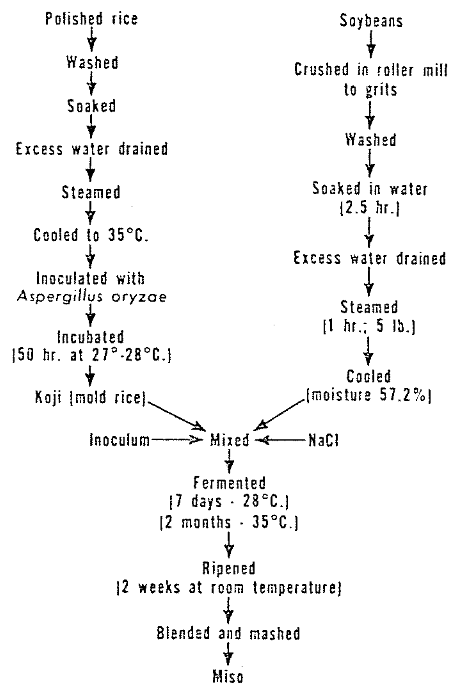

FIG. 11.1. PROCESS FOR MAKING RED MISO

The inoculated rice is then carried by closed conveyor tubes to the koji rooms which may be circular or rectangular. The rectangular rooms may be about 7 ft high and perhaps 15 ft wide and 30 ft or more in length. In the older process, the rice was placed in wooden trays. During development of the koji, temperature (35-40° C), moisture, and aeration are extremely important factors and must be rigidly controlled. Since fermentation is carried out in large rooms, contamination by bacteria is a problem. Bacterial growth is characterized by overheating, resulting in condensation of free water on the rice kernels which causes even more rapid growth of bacteria. Contamination is prevented by steaming the koji rooms, by air filtration, and by ultraviolet lamps to reduce weed microorganisms that might be present in the air introduced into koji rooms. An important factor, however, is to keep the rice kernels moist but not to permit free water on the surface of the kernels. Also during the preparation of koji, the rice is turned several times. During this stirring, the bottom rice must be brought to the top and the lumps broken apart to maintain uniform temperature, moisture, and aeration throughout the fermenting rice. In modern plants this turning of the rice is done with automatic mechanical devices. In the old process stirring was done by hand and this operation increased the amount of contamination.

To encourage mold growth, the humidity is maintained above 90%. In new plants, the air is washed and, hence, high in moisture. Ventilation must be adequate to supply sufficient oxygen and to remove the carbon dioxide.

In about 40-48 hr, the cooked rice is completely covered with white mycelium of the inoculated *A. oryzae* strains. The miso koji appears as a white felt of mycelia with the rice kernels bound together with mycelium. Harvesting is done while the koji is white and before any sporulation has occurred. At this time good koji has a pleasant smell, lacks any musty or moldy odors, and is quite sweet in taste. At this time the rice koji should be removed from the koji room, stirred, cooled, and then mixed with salt, cooked and cooled soybeans, and the yeast inoculum for the second fermentation.

Treatment of Soybeans

Simultaneous with koji production, soybeans are being prepared for fermentation. Although almost any variety may be used, manufacturers require that the soybeans should have a very light yellow, thin, and glossy seed coat; a light yellow or white hilum; a high protein content; absorb water uniformly and cook rapidly; and be large and even in size. The miso made with soybeans should be a product that is uniform in color and consistency.

Beans are cleaned, washed, and soaked in water overnight, absorbing about 1.2 times their weight of water. The beans are cooked under 10-15 lb of pressure until they are sufficiently soft to be mashed between the fingers for dark red miso but less cooking and pressure are needed for white or yellow miso. Cooking temperature and time influence color and flavor of the final product. Other factors that determine flavor and color depend on the proportion of soybeans to rice, the koji, amount of salt, and length of fermentation. Cooked soybeans are chopped or mashed. For white or light yellow miso, dehulled soybeans or soybean grits are required. A patent by Smith *et al.* (1961) covers the use of grits.

Mixing.—After cooling the beans are mixed with rice koji, salt, and water containing the inoculum. To obtain the proper moisture level, about 10% water is added with the inoculum. In the past, the inoculum was miso from a previous fermentation that contained the mixed inoculum of yeast and bacteria. Naturally, the inoculum from a previous fermentation is a selected flora of salt-tolerant yeast and bacteria capable of growing under anaerobic conditions. The dominant organisms are yeast *Saccharomyces rouxii* and certain lactic acid-producing bacteria. Actually, another yeast, *Torulopsis* sp., may also be present. In recent years, pure culture starters speed up the fermentation and reduce the influence of weed yeasts and bacteria.

The proportions of ingredients at the time of mixing, which is carried out in closed blending equipment, determine the type, color, flavor, odor, and appearance of the final product. Thus, white miso contains more rice than soybeans while darker types will contain 50-90% soybeans. Less expensive grades of miso are made from defatted soybean meal instead of whole soybeans. The true miso flavor is believed to come entirely from soybeans, whereas sweetness is derived from the sugars produced by enzymes from rice koji. White miso contains less salt (4-8%), which permits more rapid fermentation but gives the product a shorter shelf-life; red or brown miso contains 11-13% salt. The inoculum, on the other hand, is always the same regardless of the type of miso being made.

Miso contains 48-52% water. If it is less than 48%, the miso is too hard, and if more than 55%, the product becomes too soft. Naturally, moisture content greatly affects the velocity of the fermentation.

Fermentation.—After blending, the mixture is transferred to open tanks either made of wood or concrete. Weight is placed on top of the tanks to force liquid to the surface ensuring anaerobic conditions. Wooden tanks are used in the most modern plants because they can be picked up with fork lifts and moved into and out of incubator rooms. Also, they are easier to dump or unload at the time of harvest. These usually are quite large with a capacity of four tons or more. Wooden tanks last for years without damage due to moving and dumping. Since they can be moved, they may be washed easily between fermentations.

During fermentation the enzymes convert the rice into dextrin, maltose, and glucose, which serve as fermentable sugars for the yeasts and bacteria. The soybean protein is converted to peptides and amino acids. One of the chief amino acids produced is glutamic acid, which gives the delicious flavor to miso. Soybean oil is converted in part to fatty acids.

The fermentation temperature is carried out at 30°-38° C. The length of fermentation varies with the type of miso being made. For light yellow miso at least 1 month is required, whereas 3 months are necessary for red miso. The darker grades of miso, containing a high concentration of salt, may take from 3 to 12 months. These darker grades can be preserved for several months without refrigeration. The high salt concentration in these grades inhibits the growth and development of undesirable microorganisms including those which produce toxins. In some regions, miso is fermented at the temperature of locality and such miso is called "natural miso." This type requires at least one whole summer to complete the fermentation because little fermentation occurs during winter months.

Today, a revolution has occurred in the manufacture of miso, which has taken the form of automated equipment and continuous processing.

One of the big problems still remaining is the control of microorganisms because the fermentation is carried out under an open system. Thus, undesirable microorganisms can develop that can grow under anaerobic conditions and in a high salt concentration. This contamination is especially true when the fermentation and aging time is long.

According to Watanabe (1969), there is a trend towards miso being made in factories. There is a trend also towards larger factories; for instance, in 1959, there were 3000 factories while in 1965 there were 2400. The largest company has a capacity of 22,000 metric tons per year. The current price range of miso in Japan varies from 28¢ to $1.10 per kg, depending on the kind and grade of miso.

Ebine (1967) has outlined analytical methods used in the quality control during miso processing and those suitable in evaluation of the product.

SHOYU

Shoyu is the Japanese name for a dark-brown liquid, with a salty taste and sharp flavor, which is made by fermenting soybeans, wheat, and salt. It is an all-purpose seasoning agent used in preparation of foods as well as a table condiment in oriental and many other countries. The product is known as chiang-yu in China; tao-yu in Indonesia; and tayo in the Philippines. Shoyu is the only traditional oriental fermented product that has become well-known in the cookery of Western countries, and is often referred to as soy sauce. Among fermented products, chiang-yu is considered to be the most important one in China, whereas in Japan miso and shoyu are of nearly equal importance with shoyu production exceeding 1.2 million kiloliters (315 million gallons) a year.

The fermentation of shoyu is essentially a process of enzymatic hydrolysis of proteins, carbohydrates, and other constituents of soybeans and wheat to peptides, amino acids, sugars, alcohols, acids, and other low molecular compounds by the enzymes of mold, yeast, and bacteria. In addition to the fermentation technique, two other processes are followed. One is a chemical method in which acid hydrolyzes the proteins and the carbohydrates; and the other, a combination of the two. Traditionally, shoyu is made of whole soybeans; however, in recent years defatted soybean meals and flakes have taken its place. Table 11.1 indicates that 90% of the 170,000 metric tons of soybeans consumed in shoyu production in Japan per year are defatted soybean products.

Fermented shoyu.—The fermentation method involves two steps similar to that of miso fermentation except that more water is used in shoyu production and the filtered liquid, not the whole product, is consumed. The process flowsheet is shown in Fig. 11.2.

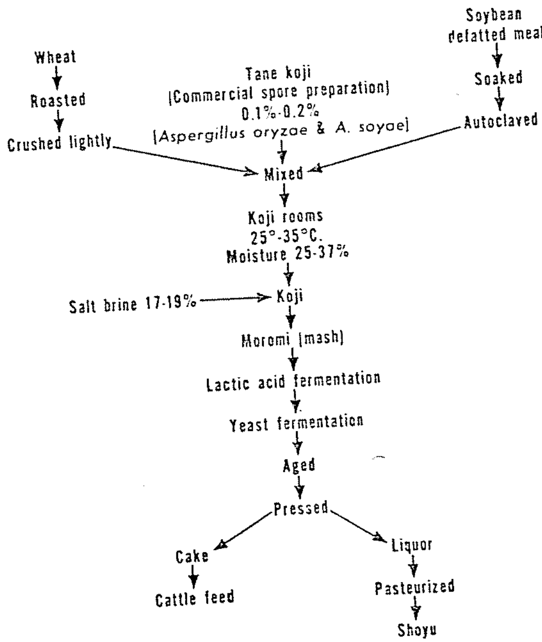

FIG. 11.2. FLOWSHEET FOR MANUFACTURE OF SHOYU

The preparation of shoyu koji from defatted soybean meals or flakes was described by Umeda *et al.* (1969). Defatted soybean products are first moistened by spraying with water amounting to about 130% soybean weight, which are then steamed at 13 lb for 45 min. Whole soybeans are usually soaked overnight, drained, and then steamed at 10 lb for several hours. After steaming and cooling, the mass is mixed with wheat that has been roasted and cracked or very coarsely ground. The proportion of soybeans to wheat may vary from one manufacturer to another. In Japan, equal amounts of soybeans and wheat are strictly observed, whereas in China, soybeans and wheat are mixed in ratios from 4:1 to 1:1. The mixture having a high ratio of soybeans to wheat produces a product rich in nitrogen and taste; however, it is difficult to make koji of superior quality from such mixtures because koji made from protein-rich and moistened substrate is easily contaminated by undesirable bacilli. The mixture is next inoculated with tane koji (*A. oryzae* and *A. soyae*) at a rate of 0.1–0.2% and then incubated at 30° C. After 24 hr of incubation, the mixture is covered with a thin white growth of mold. As mold growth continues, the temperature of the mixture could rise above room temperature to 40° C or higher. The mixture, therefore, should be turned or stirred several times during koji making to maintain uniform temperature, moisture, and aeration. The mixture should also be free of lumps to minimize bacteria propagation. As the incubation time increases, the molds continue to grow and their growth turns yellow and dark green. The moisture of the mixture gradually decreases. After about 72 hr of incubation, the shoyu koji, which has a moisture content about 26%, is ready to be harvested. In recent years, automatic koji-making processes have been developed to replace the traditional way of making koji involving wooden trays and hand mixing. The new equipment includes a continuous cooker, automatic inoculator, automatic mixer, large perforated shallow vats in closed chambers equipped with forced air devices, temperature controls, and mechanical devices for turning the substrates during incubation.

Shoyu koji of superior quality has a dark green color, pleasant aroma, sweet but bitter, vigorous mold growth, high population of yeast, low bacteria count, and strong activities of proteases and amylases.

The second step in preparation of shoyu is brine fermentation. Shoyu koji is transferred to a deep vessel in which a salt solution of 22.6% is added to make a liquid mash of about 18% of salt. A typical example of raw materials used in shoyu fermentation reported by Umeda *et al.* (1969) is as follows: defatted soybean meal, 330 kg; wheat, 337.5 kg; water, 430 liters; salt solution of 22.6%, 1200 liters. *Lactobacillus delbrueckii* and strains of yeast *Hansenula* are added to the mash. The liquid mash, or moromi as it is called by Japanese, is stirred frequently to provide enough aeration for good growth of yeast, to prevent the growth of undesirable anaerobic microorganisms, to maintain uniform temperatures, and to facilitate the removal of carbon dioxide. In modern plants, this process is carried out in large concrete vats with aeration devices. The change of temperature is said to be important for normal progress of fermentation. Therefore, shoyu fermentation in Japan usually starts in April and it will take a year to complete. In general, low-temperature fermentation gives better results; because the rate of enzyme inactivation is slow, the enzymes remain active longer (Komatsu 1968). Watanabe (1969) indicated that good quality shoyu can be obtained by 6-month fermentation when the temperature of moromi is controlled as follows: starting at 15° C for 1 month, followed by 28° C for 4 months, and finishing the fermentation at 15° C for 1 month. Takeda and Nakayama (1968) found that the fermentation time can be reduced from 1 yr to about 2 months when koji is enriched with peptidase. Steamed, defatted soybean flakes are first hydrolyzed by commercial bacterial protease at 55° C for 20 hr. and then mixed with roasted wheat to make koji. The peptidase of koji so prepared is much higher than that of conventional koji.

A perfect fermented moromi should have a bright reddish brown color, pleasant aroma, and be salty but tasty. The matured moromi is pressed to remove the liquid. The liquid or raw shoyu is then pasteurized at 65°–80° C, filtered to remove precipitates, and bottled ready for market.

Umeda (1963) and Umeda *et al.* (1969) have reported analytical results of shoyu made from whole soybeans and defatted soybean meal by 11 factories in Japan. Their average results are given in Table 11.5.

TABLE 11.5

AVERAGE COMPOSITION OF SHOYU MADE FROM WHOLE SOYBEANS AND DEFATTED SOYBEAN MEAL

	Raw Material	
Conditions	Whole	Defatted Meal
Baumé°	22.7	23.4
NaCl (%)	18.5	18.0
Total nitrogen (%)	1.6	1.5
Amino nitrogen (%)	0.7	0.9
Reduced sugar (%)	1.9	4.4
Alcohol (%)	2.1	1.5
Acidity I (ml)	10.1	14.0
Acidity II (ml)	9.8	13.6
pH	4.8	4.6
Glutamic acid (%)	1.3	1.2
Nitrogen yield (%)	75.7	73.7

A good shoyu has a salt content of about 18%. Its pH is between 4.6 to 4.8; below that the product is considered too acid suggesting acid produced by undesirable bacteria. It is also generally recognized in Japan that the quality and price of shoyu are determined by nitrogen yield, total soluble nitrogen, and the ratio of amino nitrogen to total soluble nitrogen. The nitrogen yield is the percentage of nitrogen of raw material converted to soluble nitrogen showing the efficiency of enzymic conversion. The total soluble nitrogen is a measure of the concentration of nitrogenous material in the shoyu indicating a standard of quality. A ratio of greater than 50% of amino nitrogen to total soluble nitrogen is also evidence of quality. These results can be affected by many factors such as raw materials, steaming conditions, tane koji, and brine fermentation. Technology to improve these values is constantly being sought. Defatted soybean products have proved to be as good a raw material as whole soybeans for shoyu fermentation, whereas full-fat soybean meal is not a good substitute for whole soybeans. Not only does full-fat soybean meal give a low nitrogen yield, it also results in a product having too much acid taste. Alcohol-washed meal can produce a high quality shoyu as well as a high nitrogen yield; however, the cost of alcohol-washed meal makes it impractical as a raw material.

The high salt content of shoyu prevents the growth of most microorganisms; preservatives such as *n*-butyl-*p*-hydroxy-benzoate and sodium benzoate are also used in shoyu. *n*-butyl-*p*-hydroxy-benzoate is easily hydrolyzed by enzymes produced by Aspergilli, but Hanaoka (1962) reported that enzyme activity is greatly inhibited by high salt concentration, heat treatment, and a long period of fermentation.

The chemical changes in the production of shoyu and its flavor are complicated. Yokotsuka (1960) has written a complete review on these changes. He states that of the total nitrogen, about 40-50% are amino acids, 40-50% peptides and peptones, 10-15% ammonia, and less than 1% protein. Seventeen common amino acids are present, glutamic acid and its salts being the principal flavoring constituents. The organic bases, believed to be hydrolyzed products of nucleic acids, are adenine, hypoxanthine, xanthine, guanine, cytosine, and uracil. Sugars present are glucose, arabinose, xylose, maltose and galactose; also two sugar alcohols, glycerol and mannitol. Organic acids reported in shoyu are lactic, succinic, acetic and pyroglutamic.

According to Watanabe (1969) 1 metric ton of soybeans will produce about 5 kl of shoyu. Based on this estimate, the amount (170,000 metric tons) of soybeans going into shoyu production yearly in Japan is rather high (1.2 million kiloliters). The blend of chemical shoyu with fermented shoyu in the commercial products perhaps accounts for the difference.

Chemical Shoyu.—The chemical method of making shoyu is a process of acid hydrolysis. According to Watanabe (1969), defatted soybean products or other proteinous materials are hydrolyzed by heating with 18% hydrochloric acid for 8-10 hr. After hydrolysis, the hydrolysate is neutralized with sodium carbonate and filtered to remove the insoluble materials giving a clear dark-brown liquid, or chemical shoyu. Acid hydrolysis usually results in a more complete breakdown of substrates than enzymatic hydrolysis; however, acid hydrolysis cannot perform many of the other specific reactions or interreactions of hydrolyzed products as carried out by multiple enzymes produced by molds, yeasts, and bacteria. Chemical shoyu, therefore, is a solution of amino acids and salts which is tasty and, according to oriental taste, does not possess the flavor and odor of fermented shoyu. It is often blended with fermented shoyu before being sold.

Attempts have been made to combine the chemical method, which is convenient, with the fermentation process, which produces the more desirable flavor characteristics of shoyu. First, the substrates are subjected to partial acid hydrolysis. After neutralization, the partially hydrolyzed material is mixed with moromi and the fermentation is carried out for about a month. Tenbata and Morinaga (1968) report that the taste of chemical shoyu was improved by brewing acid hydrolysates of soybeans with 3-5 volumes of moromi for 10-30 days.

NATTO

In the natural fermentation of soybeans, molds usually dominate, but natto is one of the few products in which bacteria predominate during fermentation. *Bacillus natto,* identified as *Bacillus subtilis,* is claimed to be the organism responsible for natto fermentation. Consequently, natto possesses the characteristic odor and persistent musty flavor of this organism, and is also covered with viscous, sticky polymers that this organism produces. Because of its characteristic odor, flavor, and slimy appearance, natto, even though it is well known in Japan, is not so popular nor so widely consumed as miso.

In Japan, natto is eaten with a sauce or mustard and often used for breakfast and dinner along with rice. Making natto is a simple operation and can be easily done at home. Before the microorganism was isolated, natto was made by wrapping cooked soybeans in rice straw and setting in a warm place for 1-2 days. The quality of the product is then ascertained by the stickiness of the beans and their flavor. Rice straw was credited not only in supplying the fermenting organism, but also in providing the aroma of straw, which many consumers were fond of, and in absorbing the unpleasant odor of ammonia from natto.

Many papers have been published concerning the microorganisms in natto fermentation; however, it is now well established that bacilli are the most important ones. Based on Muramatsu's account (1912), Sawamura was first to give the name of *B. natto* to 1 of the 2 bacilli that he isolated from natto. He identified the other one as a variety of *B. mes. vulgatus.* He also believed that both bacilli were required to make good natto. *B. natto* produced natto with good taste and aroma and *B. mes. vulgatus* provided the needed stickiness. But Muto (also cited by Muramatsu 1912) found that only one bacterium, which belonged to the *B. subtilis* group, was necessary for the preparation of natto. Muramatsu (1912) also made a detailed study of the three bacilli he isolated from natto. He learned that the three bacilli were similar to those isolated by Sawamura, Muto, and others. He also supported Muto's account that only 1 bacillus was essential for natto fermentation, and either 1 of his 3 bacilli was suitable for making natto. He agreed with Sawamura that the organism similar to *B. natto* Sawamura did not yield enough viscosity, but Muramatsu discovered that whenever the fermentation was carried out at high temperature (45° C), the organism produced natto with high viscosity and good taste. All three bacilli produced trypsin-like proteolytic enzymes.

In 1960, Sakurai reconfirmed that *B. natto* is aerobic, Gram-positive rod, and classified as a related strain of *B. subtilis.* There are two types of *B. natto* in the laboratory of the Food Research Institute, Ministry of Agriculture and Forestry, Tokyo, Japan. One has optimum temperature from 30° to 45° C and the other, from 35° to 45° C. He recommended that the culture known as *B. natto* SB-3010 and having optimum temperature of 30°-45° C appeared to be the one most suitable for making natto.

Pure culture fermentation has been adopted for making natto ever since the isolation of *B. natto.* Soybeans are cleaned and soaked in water for 12–20 hr, depending on the temperature, until they approximately double in weight. The soaked beans are then cooked until tender, drained, cooled to 40° C, inoculated with a water suspension of *B. natto,* and packed in a wooden box or polyethylene bag; sometimes they are wrapped in a paper-thin sheet of pine wood. The packages, which contain about 1/3 lb of cooked beans, are then placed in an incubating room at 40°–43° C for 12–20 hr. The product has a short storage life: partly because it has a moisture content of more than 50%; partly because natto is usually prepared in small-scale plants of poor quality control. Hayashi (1959A) suggested that the addition of H_3PO_4 to soaking water (0.05–0.1%) seemed to increase the storage life of the product and yet did not affect natto quality.

To improve the keeping properties of natto and broaden its uses, dry powdered natto was developed (Sakurai and Nakano 1961). Fermentation time was reduced to 6–8 hr so that the product would be more suitable for general consumption as food. After fermentation, the beans are spread out on metal trays for drying at low temperatures, either in vacuum or aeration, until the moisture content is less than 5%; then the beans are milled. Arimoto (1961) reports that powdered natto can be added to biscuits, crackers, and soup. The addition of 15% powdered natto in biscuits, 20% in crackers, and 5% in curry soup was acceptable to school children.

There are many reports concerning the changes occurring in natto fermentation. Hayashi (1959A) made one of the most comprehensive studies on natto. His data indicated that there was no change in fat and fiber contents of soybeans during a 24-hr period of fermentation but that carbohydrate almost totally disappeared. A great increase in water-soluble and ammonia nitrogen was noted during fermentation as well as during storage. The amino acid composition remained the same. Boiling markedly decreased the thiamine level of soybeans; but fermentation by *B. natto* enhanced the thiamine content of natto approximately to the same level of soybeans. Riboflavin in natto greatly exceeded that in soybeans. Vitamin B_{12} in natto was found by Sano (1961) to be higher than in soybeans.

Conflicting results on the nutritive value of natto have been published by several investigators (Arimato 1961; Hayashi 1959B; Sano 1961). They disagreed on the nutritive value of natto protein as being superior to that of boiled soybeans. But they agreed that rats fed a diet containing natto and rice grew as well as rats receiving a complete laboratory diet; whereas, rats fed a diet of boiled soybeans and rice did not grow as well as the controls. However, Hayashi found that addition of thiamine to the diet of boiled soybeans and rice corrected the deficiency; the growth of rats receiving a thiamine-enriched diet of boiled soybeans and rice was comparable to that of rats fed natto and rice. Here is further evidence that the benefits of fermented foods are manifold.

HAMANATTO

Hamanatto is the Japanese name for a product made by fermenting whole soybeans with strains of *A. oryzae*. The fermented beans are made in the vicinity of Hamanatsu from which the name of the product was perhaps derived. However, similar products are widely produced and consumed in China, the Philippines, the East Indies, and probably in other countries of the Orient. The product is known as "toushih," (which means salted beans) by the Chinese; "tao-si" by the Filipinos; and "tao-tjo" by the East Indians. In the United States, such fermented beans are often referred to as black beans because of their color.

The methods of preparing soybeans for fermentation and the composition of the brine may vary from country to country, but the essential features are similar. Soybeans are soaked and steamed until soft, drained, cooled, mixed with parched wheat flour, and then inoculated with a strain of *A. oryzae*. After incubation, the beans are packed with the desired amount of salt, spices, wine, and water and aged for several weeks or months. The finished products are blackish. They have a salty taste and their flavor resembles that of shoyu. However, they may differ in salt and moisture contents. Hamanatto is rather soft, having a high moisture content. Toushih has a much lower moisture content than that of hamanatto and, therefore, is not so soft as hamanatto. Tao-tjo tends to have a sweet taste because sugar is often added to the brine.

A typical process for making hamanatto in Japan is outlined in Figure 11.3, based on information furnished by Dr. A. Kaneko of Nagoya University, Japan. According to Dr. Kaneko, the finished product has a salt content of 13% and a moisture content of 38%.

The fermented beans can be used as an appetizer to be consumed with bland foods, such as rice gruel, or they can be cooked with vegetables, meats, and seafoods as a flavoring agent.

TEMPEH

One of the most important fermented soybean foods, originating in Indonesia, is tempe kedelee, or tempeh. This is a cake-like product made by fermenting soybeans with *Rhizopus*. When fried in oil it has a pleasant flavor, aroma, and texture. Unlike most of the other fermented soybean foods, which are usually used as flavor agents or relishes, tempeh serves as a main dish in Indonesia. Because of its unusually good and mild flavor and because of its high protein content, tempeh has been suggested as a possible source of low-cost protein.

Making tempeh in Indonesia is a household art. The procedure may vary from one household to another, but the principal steps are as follows: Soybeans are soaked in tap water until the hulls can be easily removed by hand. The dehulled soybeans are boiled with excess water for 30 min, drained, and spread for surface drying. Small pieces of tempeh from a previous fermentation are mixed with the soybeans. The inoculated beans are wrapped with banana leaves and

Whole soybeans
↓
Washed
↓
Soaked overnight
↓
Cooked 4 or 5 hr.
without pressure
↓
Cooled and held overnight
↓
Mixed with parched wheat flour
(2 soybeans 1 wheat flour)
↓
Inoculated with a short-or medium-stalked
Aspergillus oryzae
↓
Koji soaked in salt water
2.5 kg. soybeans, 650 g. NaCl, water
3.6 liters with some ginger
↓
Aged under pressure in a tank
↓
Beans removed from the liquid
↓
Sunned (dried)
↓
Hamanatto

FIG. 11.3 PROCESS FOR MAKING HAMANATTO

allowed to ferment at room temperature for one day. By this time, the beans are covered with white mycelium and bound together by mycelium as a cake, which has a pleasant odor. Traditionally, the cake, which is consumed within a day, is cut into thin slices, dipped into a salt solution, and fried in coconut oil. Sliced tempeh can be baked or added to soup.

To understand the fermentation, it is necessary to produce tempeh under scientifically controlled conditions. It was not until the late 1950's that two groups of scientists in the United States began to study the tempeh fermentation: New York Agricultural Experiment Station, Geneva, N. Y., and the Northern Regional Research Laboratory, Peoria, Ill. As a result, a pure culture fermentation method on a laboratory scale was developed. Changes in soybeans during tempeh fermentation and nutritional value of tempeh were studied in detail. The physiology and biochemistry of the tempeh mold are also being investigated.

We have received cultures of *Rhizopus* and other fungi isolated from different lots of tempeh in Indonesia and found that only *Rhizopus* could make tempeh in pure culture fermentation. Of the 40 strains of *Rhizopus* received, 25 of them are *R. oligosporus;* others are *R. stolonifer* (Ehren) Vuill, *R. arrhizus* Fischer, *R. oryzae* Went and Geerligs, *R. formosaensis* Nakazawa, and *R. achlamydosporus* Takeda. Apparently, *R. oligosporus* is the principal species used in Indonesia for tempeh fermentation. The characteristics of this species have been described by Hesseltine (1965).

Hesseltine *et al.* (1963B) have described a petri dish procedure for making tempeh in the laboratory (Figure 11.4). The preparations of soybeans for fermentation are similar to the traditional manner. Mechanically dehulled soybean grits are also suitable to make good tempeh. Since soybean grits absorb water easily, the soaking time can be reduced to 30 min. The beans are inoculated with spores of *R. oligosporus,* which have been grown on potato-dextro-agar slants at 28° C for 5-7 days. The spore suspension is prepared by adding a few milliliters of sterilized distilled water to the slant. The inoculated beans are mixed, packed tightly into petri dishes, and placed in an incubator at 30°-31° C for about 20 hr. *R. oligosporus* does not require much aeration as do many other molds; as a matter of fact, too much aeration may cause spore formation. It is therefore important to pack the petri dishes tightly; even so, some sporulation may still occur at the edge of the dish, but it will not affect the product. This procedure can also be adopted for making tempeh either in shallow wooden or metal trays with perforated bottoms and covers or in perforated plastic bags and tubes (Martinelli and Hesseltine 1964).

Steinkraus and his co-workers (1960) suggested the use of 0.85% lactic acid as soaking water. The dehulled, soaked beans are also cooked in the acid solution. This treatment would bring the pH of the beans to a range of 4.0-5.0. At this pH range, the growth of contaminating bacteria will be inhibited, but not that of the tempeh mold. We have not, however, encountered bacteria growth in our process. Because *R. oligosporus* produces an antibacterial agent (Wang *et al.* 1969); and because this organism also has the unique characteristic of fast growing, probably there is little chance for bacteria to gain ground before the tempeh fermentation is complete. Djien (1970) has further investigated this matter. He purposely inoculated with different amounts of *Escherichia coli, B. mycoides, Pseudomonas pyocyanea, Proteus spec.* or *P. cocovenenaus* along with *R. oligosporus* in making tempeh as described by Hesseltine. His results indicated that the fermentation is not intervened by the presence of inoculated bacteria. Djien commented that prefermentation during soaking or addition of acid to the soaking water may not be very important in the process of tempeh fermentation.

To prevent the loss of water-soluble substances during preparation and cooking, soybeans were treated in a minimum amount of water, just enough to soak the beans thoroughly, or were sprayed with a certain volume of water before autoclaving. But Smith *et al.* (1964) found that when this procedure was

Dehulled soybean grits
↓
Soaked (2 hr., 25°C.) ← Tap water
↓
Cooked (30 min.)
↓
Drained and cooled
↓
Inoculated ← Spore suspension of *Rhizopus oligosporus*
↓
Lightly packed in petri dishes
↓
Incubated 31°C. for 20-24 hr.
↓
Raw tempeh cake

FIG. 11.4. FLOWSHEET OF TEMPEH FERMENTATION ON A LABORATORY SCALE

followed, the tempeh showed less mold development and much sporulation. The product also had an unpleasant odor and poor flavor. The presence of a water-soluble and heat-stable mold inhibitor in soybeans was suggested by Hesseltine *et al.* (1963A). Later, Wang and Hesseltine (1965) found that the water-soluble and heat-stable fraction of soybeans also inhibited the formation of proteolytic enzymes by *R. oligosporus.*

When copra (pressed coconut cake) is substituted in the tempeh fermentation, it is then known as tempeh bongkrek. We have developed (Hesseltine *et al.* 1967) new tempeh-like products by fermenting cereal grains, such as wheat, oats, barley, or mixtures of cereals and soybeans with *Rhizopus.* Among the cultures known to make good soybean tempeh, *R. oligosporus* is the only species suitable to ferment cereal grains to make acceptable products. Other species either grow poorly on cereal grains or produce a product with unacceptable odor and taste.

As mentioned earlier, soybean tempeh is perishable and usually consumed the day it is made because the release of ammonia by enzymatic action causes the product to become obnoxious. Its shelf-life, however, can be prolonged by various methods. In Indonesia, they cut the tempeh into slices which are then dried under the sun. We found that the most satisfactory way to keep tempeh is first blanching to inactivate the mold and enzymes and then freezing. Steinkraus *et al.* (1961) developed a pilot-plant process to dehydrate tempeh by a hot air dryer at 93° C for 90–120 min. Recently, Iljas (1969) evaluated the acceptability and stability of tempeh preserved in a sealed can for ten weeks. There was no significant change in acceptability of the tempeh, when the can was sealed and immediately stored at −29° C or when the can was filled with water, steam-vacuum sealed, heat-processed at 115° C for 20 min, and stored at room temperature. However, when tempeh was first air dried at 60° C for 10 hr and then sealed in a can which was stored at room temperature, acceptability of the tempeh tended to decrease as storage progressed.

The effects of *Rhizopus* on soybeans have been studied by several investigators. A number of interesting changes occur in soybeans during fermentation. Steinkraus *et al.* (1960) found that the temperature of fermenting beans rises to above that of the incubators as fermentation progresses, but that it falls as the growth of mold subsides. The pH increases steadily presumably because of protein breakdown. After 69 hr of incubation, soluble solids rise from 13 to 28%, soluble nitrogen also increases from 0.5 to 2.0%, whereas total nitrogen remains fairly constant, and reducing substances slightly decrease probably due to this utilization by the mold. Similar changes were observed when wheat is fermented by *R. oligosporus* (Wang and Hesseltine 1966). Wagenknecht *et al.* (1961) reported that 1/3 of the total ether-extractable soybean lipid is hydrolyzed by the mold after 69 hr of incubation, and among all the fatty acids, 40% of the linolenic acid is utilized by the mold. Niacin, riboflavin, pantothenic acid and vitamin B_6 contents of soybeans increase after fermentation, whereas thiamine slightly decreases (Roelofsen and Talens 1964). Wang and Hesseltine (1966) also noticed in fermenting wheat with *R. oligosporus* that the amount of niacin and riboflavin of the wheat tempeh greatly exceeds that of wheat alone, while thiamine appears to be less. Apparently, *R. oligosporus* has a great synthetic capacity for niacin, riboflavin, pantothenic acid, and vitamin B_6, but not for thiamine.

Although free amino acids in tempeh increase during fermentation, the amino acid composition of soybeans is not significantly changed by fermentation (Stillings and Hackler 1965). Perhaps the amount of mycelial protein present in tempeh is not high enough to alter greatly the amino acid composition of the soybeans, nor does the mold depend upon any specific amino acid for growth as suggested by Sorenson and Hesseltine (1966).

The nutritional value of fermented products has always been a controversial subject; it is no exception in regarding the nutritional value of tempeh. Indonesians consider tempeh to be a nourishing and easily digestible food. Van Veen and Schaefer (1950) observed beneficial effects of tempeh on patients with dysentery in the prison camps of World War II, and they suggested that tempeh was much easier to digest than soybeans. However, animal feeding experiments have not substantiated this conclusion (Hackler *et al.* 1964; Smith *et al.* 1964; Murata *et al.* 1967; Wang *et al.* 1968). Nevertheless, the superior nutritive value of tempeh over untreated soybeans has been noted by György (1961) on animals fed low protein diets. His results seem to resemble those obtained with animals fed antibiotics added to their protein source. Recently, we found that *R. oligosporus* indeed produces an antibacterial agent during tempeh fermentation as well as in submerged culture (Wang *et al.* 1969). The compound is especially active against some Gram-positive bacteria including both microaerophilic and anaerobic bacteria; e.g., *Streptococcus cremoris, B. subtilis, Staphylococcus aureus, Clostridium perfringens* and *C. sporogenes.* The compound contains polypeptides having high carbohydrate content. Its activity is not affected by pepsin or *R. oligosporus* proteases, and is slightly decreased by trypsin and peptidase. It is, however, rapidly inactivated by pronase.

It is well established that antibiotics, in addition to minimizing infections, elicit growth-stimulating effects in animals especially those whose diets are deficient in any one of several vitamins, or proteins, or some growth factors. Oriental people are constantly exposed to overwhelming sources of infection and their diets are frequently inadequate. The finding of antibacterial agents produced by *R. oligosporus,* therefore, offers a possible clearer understanding of the true value of tempeh in the diet of Indonesians, and perhaps of fermented foods in the diets of all Orientals. Furthermore, these antibacterial agents would minimize the bacterial contamination during tempeh fermentation.

Of the 40 strains of tempeh mold maintained at the Northern Regional Research Laboratory, *R. oligosporus* NRRL 2710 is a typical representative. Sorenson and Hesseltine (1966) studied carbon and nitrogen utilization by NRRL 2710. They found that such common sugars as glucose, fructose, galactose, and maltose provide excellent growth of the mold; whereas the soluble carbohydrates of soybeans–i.e., stachyose, raffinose, and sucrose–are not utilized by the mold as a sole source of carbon. On the other hand, various vegetable oils can be substituted for sugars as sources of carbon. The strong lipase activity and utilization of linolenic acid by mold found in tempeh fermentation (Wagenknecht *et al.* 1961) would strongly suggest that lipid materials are the primary sources of energy. Sorenson and Hesseltine also disclosed that ammonium salts and some amino acids, such as proline, glycine, aspartic acid, and leucine, are excellent sources of nitrogen for *R. oligosporus,* but that the mold does not depend on any specific amino acid for growth.

Strains of tempeh molds produce various amounts of amylase, pectinase, lipase, and proteases. Although strains of *R. oryzae* produce high amounts of amylase, *R. oligosporus* forms little or no amylase. Since starch is seldom found in mature soybeans, it is not particularly important that this species produces amylase during tempeh fermentation. Certain species of *Rhizopus* are known to be active pectinase producers. Of strains suitable for tempeh fermentation, *R. arrhizus* NRRL 1526 appears to produce the highest amount of pectinase. All the strains of *R. oligosporus* have little or no pectinase activity (Hesseltine *et al.* 1963B). Lipase is also produced by molds in tempeh fermentation (Wang and Hesseltine 1966; Wagenknecht *et al.* 1961). Wagenknecht *et al.* (1961) found that fatty acids are liberated by hydrolysis of soybean lipids, but there is no further subsequent utilization of these fatty acids. They concluded that either the mold does not possess the enzyme systems to metabolize these fatty acids or these fatty acids are not permeable to the cytoplasmic membrane of *Rhizopus*. Proteases are, perhaps, much more important enzymes in tempeh fermentation. The ability of *Rhizopus* to produce proteolytic enzymes varied greatly between different strains of the same species as well as between species (Wang and Hesseltine 1965). The proteolytic enzyme systems have optimal pH at 3.0 and 5.5, with the pH 3.0 type predominating in submerged cultivation and pH 5.5 type predominating in tempeh fermentation. The enzymes are stable at pH 3-6 and have high milk-clotting activity.

SUFU

Sufu is a phonetic rendition of the Chinese words, putrid bean curd. It is also known as "fu-ju" or "tou-fu-ju" by many Chinese, "bean cake" by Chinese grocers of this country, and "Chinese cheese" by many scientists. Many more translated names can be found in English literature (Wang and Hesseltine 1970). Indeed, these various names give a good description of the product. Sufu, therefore, is a soft cheese-type product and made from cubes of soybean curd (tofu) by the action of microorganisms. Sufu has been widely consumed as a relish by all segments of the Chinese people and has been manufactured in China long before the Ching Dynasty. Chao of Vietnam, tahuri of the Philippines, and taokoan of the East Indies perhaps are similar to sufu. But no comparison will be made because of the sparse literature on similar products from other countries.

The process of making sufu was considered a natural phenomena. Not until 1929 was a microorganism believed to be responsible for sufu fermentation isolated and described by Wai (1929). He identified the microorganism to be an undescribed species of *Mucor* and proposed the name *Mucor sufu.* Wai also thought that this fungus inhabited originally on rice straw because rice straw was always used to cover the tofu cubes for fermentation in the traditional way. Almost 40 yr later, Wai (1968) reinvestigated the microorganism in sufu fermentation and as a result a pure culture fermentation for making sufu was developed. Wai and his co-workers collected molds from several plants in Taiwan and Hong Kong. They consistently obtained strains of *Actinomucor elegans.* However, strains of *M. hiemalis* and *M. silvaticus* were isolated from homemade sufu. We have received a number of cultures reported to have been isolated from sufu fermentation of various factories and found they all belong to the genus of *Mucor* or a related genus, *Actinomucor.*

In obtaining good quality fermented products, the fungus for sufu fermentation must have white or yellowish-white mycelium to warrant an attractive appearance. The texture of mycelial mat should also be dense and thick so that a firm film will be formed over the surface of the fermented tofu cubes to prevent any distortion in its shape. It is also important that the organism elaborate enzyme systems having high proteolytic activity because the mold grows on a protein-rich medium. Furthermore, the mold growth should develop neither a disagreeable odor nor an astringent taste. Wai and his co-workers confirmed that

A. elegans, M. hiemalis, M. silvaticus and *M. subtilissimus* possess all these characteristics and can produce sufu having good quality. But they indicated that *A. elegans* is the best 1 among the 4 molds for sufu fermentation and is the 1 adopted commercially.

Three steps are normally involved in making sufu: preparing tofu, molding, and brining (Fig. 11.5). To make tofu, soybeans are first washed, soaked overnight, and then ground with water. The finely ground mixture is strained through a coarse cloth to separate the soybean milk from the insoluble residue. After the soybean milk is heated to boiling, calcium or magnesium sulfate is added to coagulate the proteins. The coagulated milk is then transferred into a cloth-lined wooden box and pressed with weight on top to remove whey. A soft, but firm cake-like curd (tofu) forms. Tofu has a bland taste and a high content of water (about 90%). It can be consumed directly and is so eaten extensively throughout the Far East. But the water content of tofu for making sufu is lower than that of tofu consumed directly; otherwise, it is likely to be spoiled by bacterial growth.

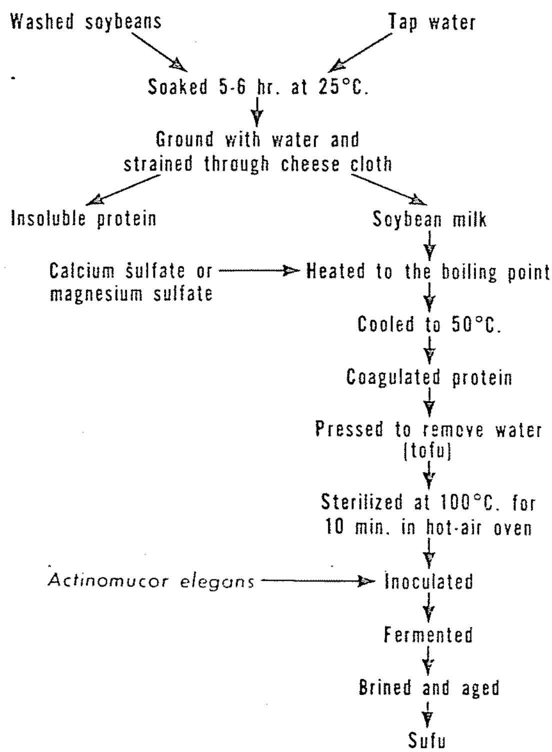

FIG. 11.5. FLOWSHEET FOR THE PREPARATION OF SUFU

According to Wai, tofu for making sufu has a water content of 83%; insoluble protein, 9.1%; soluble protein, 0.4%; and lipid, 4.0%. To prepare for molding, tofu is cut into small cubes of about 2 X 2 X 4 cm. The cubes are immersed in an acid-saline solution of 6% sodium chloride plus 2.5% citric acid for 1 hr and then subjected to hot air sterilization at 100° C for 15 min. This treatment prevents the growth of contaminating bacteria but does not affect the growth of fungi needed in making sufu. Tofu cubes should be separated from one another in a tray with small openings in the bottom and top to facilitate the circulation of air, because mycelia are required to develop on all sides of the cubes. After cooling, the cubes are then inoculated over their surface with pure culture of an appropriate fungus grown on filter paper impregnated with a culture solution. The inoculated cubes are incubated at 20° C or lower for 3-7 days depending on the culture. The freshly molded cubes, known as pehtze, have a luxurious growth of white mycelium and no disagreeable odor. The pehtze has a water content of 74%; insoluble protein, 10.9%; soluble protein, 1.3%; and lipid, 4.3% as reported by Wai.

The last step in making sufu is brining and aging. The pehtze are placed in various types of brining solutions depending on the flavor desired. The most common brine would be one containing 12% sodium chloride and rice wine amounting to about 10% of ethyl alcohol. The immersed cubes are allowed to age for about 40-60 days. The product is then bottled with the brine, sterilized, and marketed as sufu.

Other additives, either to give color or flavor, are frequently incorporated into the brine. Red rice and soy mash are added to the brine to make a red product known as "red sufu." Fermented rice mash can be added to the brine so that the product has a more alcoholic fragrance; tsui-fang, which means drunk sufu, is so made. The addition of hot pepper to the brine would make hot sufu. Rose sufu is one aged in brine containing rose essence. Therefore, the taste and aroma of sufu, in addition to its own characteristics, can be easily enhanced or modified by changing the ingredients of the brining solution.

Changes occurring during the aging process were also studied by Wai and his co-workers. After 30 days of aging at room temperature, total soluble nitrogen increased from 1.00 to 2.74% and total insoluble nitrogen reduced from 7.89 to 6.05%, while total nitrogen changed slightly. The soluble nitrogenous compounds were reported to consist of soluble proteins, peptides, and amino acids, including aspartic acid, serine, alanine, leucine/isoleucine, and glutamic acid. Lipids in pehtze were also partially hydrolyzed through the aging period. Free fatty acids increased from 12.8 to 37.1% and total lipids remained unchanged.

Tofu has a high content of protein and lipids, 55 and 30%, respectively. Therefore, it is expected that the molds would produce high activities of proteolytic and lipolytic enzymes. The proteolytic enzymes elaborated by *M. hiemalis* 28 NRRL 3103 (Wang 1967) and *A. elegans* NRRL 3104 have been studied at the Northern Regional Research Laboratory. *M. hiemalis* grown in soybean medium produces protease having optimal pH 3.0–3.5, whereas *A. elegans* produces proteolytic enzymes having optimal pH at 3.0, 6.0, and 9.0. However, enzyme activity is very low in culture filtrates unless sodium chloride is added to the medium. Further work indicated that the enzyme is loosely bound to the mycelial surface, possibly by ionic linkage and can be easily eluted by sodium chloride or other ionizable salts. Apparently, sodium chloride in the brining solution not only retards mold growth and imparts a salty taste to the final product, but also releases the mycelium-bound proteases, which, in turn, penetrate the pehtze and act on the protein.

Lipolytic enzymes produced by sufu molds were studied by Wai (1968). He found that the enzymes can hydrolyze glycerol esters of fatty acids, as well as such phospholipids as lecithin, which is high in soybeans. A mixture of phospholipases, perhaps, is produced by the molds because choline and fatty acids, stearic acid, palmitic acid, and linoleic acid were formed in the reaction mixture when lecithin was the substrate.

In addition to its salty taste, sufu has its own characteristic flavor. Since the composition of substrate in sufu fermentation is rather a simple one, it is likely that the hydrolytic products of proteins and lipids provide the principle flavor constituents of sufu. The added wine, fermented rice mash, anise, or any synthetic flavor to the brining solution contributing esters, organic acids, sugars, and alcohols further enhance the flavor and aroma.

Traditionally, sufu is consumed directly as a relish or is cooked with vegetables or meats. Either way, sufu adds a zest to the bland taste of a rice-vegetable diet. Because sufu is a cream cheese-type product and has a mild flavor, it would be suitable to use in Western countries as a cracker spread or as an ingredient of dips and dressings.

NEW SOYBEAN PRODUCTS MADE BY FERMENTATION

Cheese-type Products

Soybean milk and soybean curd or tofu to the people of Asia have the same importance as cow's milk and cheese to the people of dairy countries. The relationship of soybean curd to soybean milk is like that of cottage cheese to cow's milk, except that the curdling of soybean milk is traditionally accomplished by the addition of calcium salts, or occasionally by acid. Asiatic people have preferred the method of salt precipitation for making curd. Not only does the salt precipitation yield a product having acceptable texture, but also the salt-precipitated curd serves as a good source of calcium. Unlike most types of cheese, soybean curd is usually consumed without the ripening process carried out by microorganisms. Sufu, as described, is the only traditional fermented soybean product made by a curdling process and a ripening process carried out with a mold.

In recent years, attempts have been made to develop a new cheese-type product from soybean milk through the use of cultures and technology employed in the making of cheese from cow's milk. Although these new products have not become available, these studies have resulted in several publications and patents. These studies show that in addition to the method of salt precipitation, the curdling of soybean milk can be brought about by the acid produced by lactic acid bacteria. The ripening process can be carried out by enzyme preparations, bacteria, or mold.

Hang and Jackson (1967) made a cheese from soybean milk using a cheese starter, *Streptococcus thermophilus.* Although the product had a clean and fresh flavor, the process did not change the substrate other than that the acid produced by the bacteria caused soybean milk to curdle. During the 63 days of ripening, the starter bacteria gradually disappeared; other lactobacilli of primary importance in cheesemaking were not found. The water-soluble nitrogen remained constant throughout the ripening process, a condition indicating the lack of proteolysis. However, they found that the addition of rennet extracts and skim milk to the soybean milk and starter stimulated proteolysis and yielded a product having a clean and mild flavor.

Kenkyusho (1965) of Japan used *S. faecalis* as a starter to produce a cheese-like product. Soybean milk was first supplemented with casein, glucose, butter fat, and vegetable oil. The mixture was emulsified, heated, and then treated with *S. faecalis,* rennet extract, and calcium chloride. The resulting curd was then treated according to the conventional method of cheese making.

Soybean or defatted soybean powder was also used to make a cheese-like product. The powder was mixed with water and then treated with takadiastase or other digestive enzymes (Nihon Koyu Kogyo Co. 1965) or inoculated with mold (Kikkoman Shoyu Co. 1965).

According to Obara of Tokyo University of Education, Japan, no acceptable product can be obtained from soybean milk by the conventional cheese-making process. Obara (1968) treated the curd obtained by salt precipitation with proteolytic enzymes. After aging, the finished product had a good flavor and texture. His procedure is outlined in Fig. 11.6. He found that calcium sulfate at the concentrations of 0.03–0.04 N was the best salt to curdle soybean milk. The starter culture was a mixture of *S. cremoris* and *S. lactis* (1:1). Among the proteolytic enzymes tested, papain at a concentration of 0.394% in respect to the protein content gave the best result. The product had an extremely smooth texture and desirable taste. Pronase and bioprase were also considered to be suitable. Trypsin and molsin, however, were unsatisfactory because of their poor digestion ability and the inferior taste of the product.

Fermented Soybean Milk

Soybean milk has a long history as a popular beverage in Asia, but fermented soybean milk has never been a traditional one. In recent years, attempts have

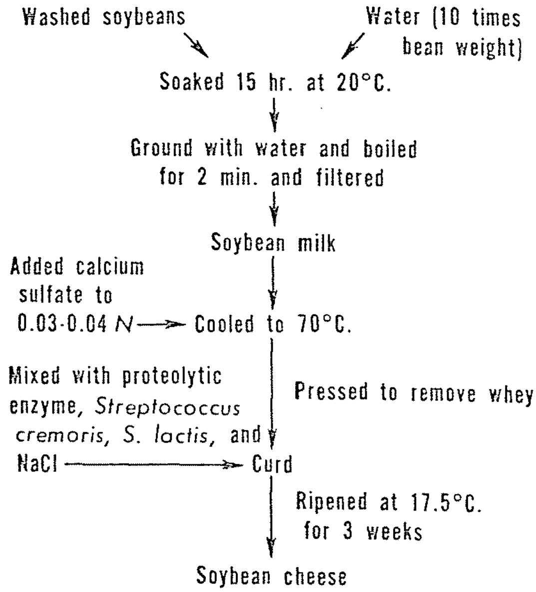

FIG. 11.6. FLOWSHEET FOR THE PREPARATION OF SOYBEAN CHEESE

been undertaken to develop fermented soybean milk by the same cultures employed in making fermented cow's milk.

In Japan, when soybean milk was subjected to yogurt-type fermentation (Ariyama 1963), the resulting product had higher protein and mineral contents than that of yogurt made from cow's milk. To prepare soybean milk with a high-protein content, Ariyama suggested that the soybeans be first steamed, then soaked and ground with 0.1% NaOH solution, and filtered. According to Ariyama, the use of 0.1% NaOH to extract soybeans yielded a soybean milk having a higher protein content than that of soybean milk obtained by water extraction. Before fermentation, the milk was neutralized with hydrochloric or citric acid and supplemented with 15% cane sugar. The enriched soybean milk was then boiled, cooled, and inoculated with *Lactobacillus bulgaricus*. After incubating at 37–43° C for 4–6 hr, the soybean milk coagulated into a yogurt-like product. Ariyama's results indicate that soybean milk yogurt has a protein content of 9.8% as compared to 3.4% in yogurt made from cow's milk.

At the Northern Regional Research Laboratory, acidophilus-type soybean milk has been prepared. Freshly prepared soybean milk was sterilized at 120° C for 30 min, cooled, and inoculated with *L. acidophilus* NRRL B-629 and incubated at 37° C for 24 hr. The resulting fermented soybean milk tasted good after addition of sugar and vanilla flavoring.

An Ontjom-type Product

Ontjom is an Indonesian food made by fermenting peanut press cake with strains of mold belonging to *Neurospora.* A culture isolated from ontjom was identified as *Neurospora sitophila* and is now maintained in our USDA–ARS Culture Collection designated as NRRL 2884. In Indonesia, the ontjom fermentation is carried out much like tempeh and the product is also eaten much like tempeh. Coconut press cake and residue from soy milk product are occasionally used as substrates, but dehulled soybeans are not known to be used for ontjom fermentation in Indonesia.

Steinkraus *et al.* (1965) studied the ontjom mold to ferment soybeans. Their fermentation yielded a tempeh-like product having an acceptable flavor and texture. The dehulled beans were soaked for 17–18 hr at 25° C in water acidified with 85% lactic acid on a 1.5–2.0% vol/vol basis. The soaked beans were cooked for 90 min at 100° C, drained, cooled, and inoculated. They used a culture of *Neurospora* isolated from ontjom obtained from Indonesia. The inoculum was prepared by growing the mold on sterilized soybeans which were then freeze dried and ground. One gram of the inoculum was used to inoculate 1 kg of cooked beans. The inoculated beans were packed approximately 1 in. thick in a covered stainless steel pan (10 × 14 × 2-1/2 in.) and incubated at 30° C. After 35–40 hr incubation, the beans were bound together by the mold mycelia into a cake-like product similar to tempeh. When the bean cake was sliced and deep-fat fried, it had an almond-like characteristic flavor which was not quite like that of tempeh.

The studies of Steinkraus *et al.* also indicated that the ontjom mold, *Neurospora* sp., had a lower maximum growth temperature than does *R. oligosporus* and that the fermentation cannot be carried out at a temperature above 32° C. During fermentation, the pH gradually rose. The total solids and total nitrogen remained fairly constant, whereas soluble solids and soluble nitrogen progressively increased. Thus, the changes in soybeans during fermentation were similar to those in tempeh.

FUTURE OF FERMENTED SOYBEAN FOODS

If we concede that more and more people will have to use nonanimal protein, then what direction should the development of soybean foods and particularly soybean food fermentations take? The mistake is often made that what we in the West believe is acceptable food, should also be acceptable in the rest of the world. There is a better alternative to this approach. Each country or cultural group has developed a preference for certain food flavors, textures, mouth feel, and appearance. Why not recognize and accept these cultural preferences and develop foods on scientific grounds that do not alter the target people's preferences? Recently, we heard that bulgur being imported into the East Indies is not being used as it was intended but rather mixed with soybeans and then fermented into cakes with the tempeh mold. The product now is acceptable because it takes on a form familiar to the people. Native foods should be examined in detail and modern scientific methods used to develop a food technology on the product. Hence, acceptance would be assured by the native people toward which the market is aimed. If research were developed in this direction, problems of microbial spoilage, retention of vitamins, uniformity of product, and lowered cost could be solved. This concept applies as well to nonfermented foods. Therefore, the goal of fermented soybean food research for developing countries should be aimed at the preparation of a native food by modern technology to give a uniform product which is cheap, nutritious, free of dangerous microorganisms, and completely familiar to the native population.

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